

RESTRICTED NUCLEOCYTOPLASMIC RELATIONSHIP IN  
ACTIVATION OF T AND B LYMPHOCYTES\*

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T and B lymphocytes stimulated by mitogens or specific antigens have been shown to undergo proliferative responses. These proliferations are triggered by the binding of the various ligands with cell surface receptors. Signals given from the cell surface receptor are transferred to the nucleus through a cytoplasmic signal-transmitting system. However, the exact mechanisms for the activation of lymphocyte nuclei have not yet been established. We recently described a method for separating mouse splenic lymphocytes into karyoplasts and cytoplasts (1) as well as a method for transferring lymphocyte nuclei (karyoplasts) from one cell type into another cell type using polyethyleneglycol-mediated cell fusion (2, 3). Our previous study showed that fusion of lethally irradiated murine splenic lymphocytes with lymphocyte nuclei (karyoplasts) could save the irradiated cells from death in vitro, and karyoplasts injected into the irradiated cells could be activated by mitogens, such as concanavalin A (Con A) or lipopolysaccharides (LPS), in the reconstituted cells. To analyze the nuclear-cytoplasmic relationship in such reconstituted cells, we examined, in the present study, whether or not T cell nuclei can be stimulated in the B cell cytoplasmic compartments as well as in the T cell cytoplasm or B cell nuclei can be stimulated in the T cell cytoplasmic compartment. Nuclei (karyoplasts) purified from T or B cells were introduced into x-ray-irradiated B or T cells, and the reconstituted hybrid cells were stimulated with T cell mitogen, Con A, or B cell mitogen, LPS. The present report shows that nuclei of T cells can be activated in the Con A-stimulated T cell cytoplasts but not in the LPS-stimulated B cell cytoplasts. On the other hand, nuclei of B cells were activated in the LPS-stimulated B cells but not in the Con A-stimulated T cell cytoplasm. These data suggested that the specific interaction between cytoplasm and nucleus plays an important role in the activation of lymphocyte nuclei in addition to the interaction of mitogens with their specific cell surface receptors.

**Materials and Methods**

*Enucleation of Murine Splenic Lymphocytes.* Lymphocytes were enucleated by centrifugation through a Ficoll density gradient in the presence of cytochalasin-B (CB), as described before (1), by a modification of the procedure of Wigler and Weinstein (11). Mouse splenic lymphocytes were suspended in 12.5% Ficoll-400 (Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, NJ) in Eagle's minimum essential medium (MEM) containing 10  $\mu$ g/ml CB and 0.5% dimethylsulfoxide (DMSO) and layered on Ficoll density gradients in cellulose nitrate tubes. Ficoll gradients were prepared in Eagle's MEM containing 10  $\mu$ g/ml of CB and 0.5%

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DMSO and carefully layered in the following: 4 ml of 25%, 4 ml of 17%, 1 ml of 16%, 1 ml of 15%, and 4 ml of 12.5% Ficoll. The cells on the Ficoll gradients were centrifuged at 23,000 rpm in a Beckman SW27 swinging bucket rotor (Beckman Instruments, Inc., Fullerton, CA). After centrifugation, almost all cells were found to be separated into karyoplasts (nuclei) and cytoplasts (enucleated cells). Karyoplasts were further purified by 1 *g* velocity sedimentation using bovine serum albumin gradients (1).

**Lymphocytes.** Splenic lymphocytes were prepared from C3H/HeN mice. Purified T cells were obtained by passing spleen cells through a nylon-wool column. More than 95% of the cells of the T cell fraction expressed Thy-1.2 on the surface. Purified B cells were prepared as follows. The spleen cells were first incubated on plastic dishes (3003; Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, CA) for 2 h at 37°C in a CO<sub>2</sub> incubator. Nonadherent cells were placed on goat anti-mouse Fab-coated dishes for 60 min at 4°C. After washing out nonadherent cells, the adherent cells were recovered. Surface Ig-positive cells accounted for >90% of this fraction.

**Cell Fusion.** X-ray (2,000 rad) irradiated cells ( $1-2 \times 10^6$ ) and karyoplasts were mixed at a ratio of 1:5 to 1:10 in a 12-ml conical tube and centrifuged at 1,000 rpm for 10 min. Four drops of 50% polyethyleneglycol (Koch-Light, 6,000 mol wt) supplemented with 5 µg/ml poly-L-arginine and 10% DMSO (2) were added to the pellets, and the mixtures were incubated for 1 min at room temperature. The reaction was stopped by addition of 10 ml of prewarmed MEM. Cells were suspended in RPMI 1640 medium containing 10% FCS, 2 mM L-glutamine, and  $5 \times 10^{-5}$  M 2-mercaptoethanol.

**Mitogens.** Bacterial LPS was *Escherichia coli*:B8 (Difco Laboratories, Detroit, MI). Con-A was purchased from Pharmacia Fine Chemicals, Div. of Pharmacia Inc.

## Results and Discussion

Murine spleen cells are almost completely enucleated in suspension after centrifugation in a discontinuous Ficoll gradient in the presence of CB (1). In the present study, nuclei (karyoplasts) fractions were prepared from nylon-wool-purified T cells or from anti-immunoglobulin-coated dish-purified B cells and further purified by 1 *g* velocity sedimentation in gradients of bovine serum albumin. Purity of karyoplasts was examined by their DNA and protein contents, as described before. Whole spleen cells, T cells, or B cells from normal C3H/HeN mice were irradiated by 2,000 rad x-rays. The karyoplasts and irradiated cells were fused by 50% polyethyleneglycol supplemented with poly-L-arginine and DMSO, as described previously (2). After fusion, cells were stimulated with Con A or LPS, and [<sup>3</sup>H]thymidine uptake into the DNA of reconstituted cells was measured 72 h after culture. Data are summarized in Tables I and II. Karyoplasts of T and B lymphocytes were not viable for longer than 30 h in vitro and did not respond to Con A or LPS, resulting in no incorporation of [<sup>3</sup>H]thymidine into the DNA. X-ray-irradiated spleen cells and x-ray-irradiated T or B cells also died during culture and did not respond to any mitogen. Simple mixtures of karyoplasts and irradiated lymphocytes could not respond to any mitogens, and most of the cells were dead by the end of culture. However, in the reconstituted hybrid cells obtained from fusion between T cell karyoplasts and irradiated T cells, [<sup>3</sup>H]thymidine incorporation into DNA was observed when they were cocultured with Con A, but they were not responsive to LPS. The reconstitution of heavily irradiated T cells with T karyoplasts seemed to prevent cell death because both viability and uptake of [<sup>3</sup>H]thymidine were very similar to nonirradiated T lymphocytes. Fusion of T karyoplasts with irradiated whole spleen cells restored the background [<sup>3</sup>H]thymidine uptake into the cells, and the hybrids responded to Con A as they normally did but failed to respond to LPS. On the other hand, the reconstituted hybrid cells formed

TABLE I  
*T Cell Cytoplasmic Compartments Are Necessary for Activation of T Cell Nuclei*

Cells	Fusion	Stimulated with		
		Medium	LPS (10 µg/ml)	Con A (1 µg/ml)
Whole spleen cells		5,657 ± 1,016*	114,220 ± 2,839	197,800 ± 4,032
T cells		5,199 ± 265	4,072 ± 191	153,113 ± 1,543
B cells		3,083 ± 352	90,509 ± 189	2,770 ± 304
Irradiated spleen cells		64 ± 9	47 ± 6	75 ± 18
Irradiated B cells		47 ± 6	41 ± 10	61 ± 25
Irradiated T cells		71 ± 21	43 ± 9	41 ± 6
T karyoplasts		57 ± 5	43 ± 9	44 ± 11
Irradiated T + T-karyoplasts‡	-	54 ± 3	81 ± 25	77 ± 12
Irradiated T × T-karyoplasts§	+	10,159 ± 948	9,329 ± 873	175,203 ± 3,458
Irradiated spleen + T-karyoplasts‡	-	95 ± 35	66 ± 19	55 ± 7
Irradiated spleen × T-karyoplasts§	+	10,083 ± 465	8,924 ± 138	188,733 ± 6,861
Irradiated B + T-karyoplasts‡	-	61 ± 21	67 ± 28	35 ± 12
Irradiated B × T-karyoplasts§	+	8,414 ± 103	8,978 ± 643	8,764 ± 794

Whole spleen cells, purified T cells, or purified B cells were irradiated at 2,000 rad. Whole spleen cells irradiated at 4,000 rad were used as the macrophage source. Irradiated cells and karyoplasts were fused with 50% polyethyleneglycol supplemented with 5 µg/ml poly-L-arginine and 10% DMSO. Spleen cells ( $2 \times 10^5$  per well), karyoplasts ( $1 \times 10^6$  per well), or cells containing the hybrids ( $2 \times 10^5$  per well) were cultured in the presence or absence of mitogen for 72 h and pulsed with 0.5 µCi of [ $^3$ H]thymidine for the last 4 h of culture. Irradiated spleen cells ( $5 \times 10^4$  per well) were added to T cells, B cells, karyoplasts, or hybrid cells as a source of macrophages.

\* Counts per minute (mean ± SD).

‡ Simple mixture of irradiated T cells and karyoplasts of T cells.

§ Irradiated T cells and karyoplasts were fused with fusion reagents.

by fusion of T karyoplasts with irradiated B cells were not stimulated with either Con A or LPS, although background [ $^3$ H]thymidine uptake was recovered, suggesting that the reconstituted cells were mostly viable in culture but did not display responsiveness to either Con A or LPS. LPS responsiveness of irradiated cells was restored in the hybrids between irradiated B cells and B cell karyoplasts, as shown in Table II. The introduction of B karyoplasts into irradiated T cells, however, did not restore the responsiveness of the cells to either LPS or Con A. Furthermore, fusion of B karyoplasts with irradiated whole spleen cells could restore the DNA synthetic response to LPS but not to Con A. As previously described (2), the reconstitution of irradiated whole splenic lymphocytes with karyoplasts taken from whole spleen cells could restore the responsiveness to both mitogens. There was no difference in the fusion efficiency (10–20% of splenic lymphocytes were found to contain two nuclei) or in the cell viability of hybrids between the various combinations of karyoplasts and irradiated cells. Peak response of [ $^3$ H]thymidine uptake was also seen on the same day (day 3) with each hybrid cell. Treatment of either irradiated cells alone or karyoplasts alone with polyethyleneglycol did not restore any mitogenic response of these cells. As previously reported (2), we failed to obtain the proliferative response of the reconstituted cells

TABLE II  
*B Cell Nuclei Are Activated in LPS-stimulated Cytoplasm but Not in Con A-stimulated Cytoplasm*

Cells	Fusion	Stimulated with		
		Medium	LPS (10 $\mu\text{g}/\text{ml}$ )	Con A (1 $\mu\text{g}/\text{ml}$ )
Whole spleen cells		3,650 $\pm$ 206*	38,354 $\pm$ 2,028	25,874 $\pm$ 1,736
B cells		4,601 $\pm$ 233	51,959 $\pm$ 1,932	6,295 $\pm$ 1,078
Irradiated spleen cells		565 $\pm$ 28	627 $\pm$ 51	599 $\pm$ 38
Irradiated B Cells		550 $\pm$ 28	882 $\pm$ 408	571 $\pm$ 38
B karyoplasts		575 $\pm$ 32	848 $\pm$ 151	602 $\pm$ 20
Irradiated B cells + B karyoplasts	-	156 $\pm$ 4	388 $\pm$ 136	159 $\pm$ 10
Irradiated B cells $\times$ B karyoplasts	+	3,433 $\pm$ 292	47,027 $\pm$ 3,239	3,708 $\pm$ 105
Irradiated spleen cells + B karyoplasts	-	190 $\pm$ 12	293 $\pm$ 27	208 $\pm$ 18
Irradiated spleen cells $\times$ B karyoplasts	+	1,622 $\pm$ 173	17,602 $\pm$ 351	1,328 $\pm$ 232
Irradiated T cells $\times$ B karyoplasts	+	2,103 $\pm$ 26	2,245 $\pm$ 166	2,191 $\pm$ 199
B karyoplasts only	+	155 $\pm$ 5	158 $\pm$ 29	189 $\pm$ 25
Irradiated spleen cells only	+	174 $\pm$ 29	168 $\pm$ 49	183 $\pm$ 46

Purified B lymphocytes and karyoplasts were prepared as described in Materials and Methods. 2,000 rad irradiated cells ( $1 \times 10^6$  cells) and karyoplasts ( $1 \times 10^7$  nuclei) were fused with polyethyleneglycol supplemented with poly-L-arginine and DMSO. Purified B cells, karyoplasts, hybrid cells, or irradiated B or T cells were cultured with heavily irradiated (4,000 rad) spleen cells in the presence of mitogen for 72 h. Cultures were set up in triplicate and pulsed with 0.5  $\mu\text{Ci}$  of [ $^3\text{H}$ ]thymidine 4 h before harvest.

\* Counts per minute (mean  $\pm$  SD).

when the karyoplasts had been heavily irradiated, indicating that the DNA synthetic responses observed in the present study were due to the fused karyoplasts and not to the nuclei of irradiated cells.

The data described above suggested that nuclei of T lymphocytes could be activated in the irradiated and Con A-stimulated T lymphocytes but not in the irradiated and LPS-stimulated B lymphocytes. Accordingly, nuclei of B lymphocytes could be activated in the irradiated, LPS-stimulated B cells but not in the Con A-stimulated T cells. In other words, T cell nuclei might require specific signals from cytoplasmic compartments of Con A-stimulated T cells. Signals given from LPS-stimulated B cells might not be appropriate to stimulate T cell nuclei. Conversely, cytoplasmic factors of LPS-stimulated B cells could activate nuclei of B cells but not those of T cells.

The reconstituted cells obtained by Sendai virus-induced fusion of cytoplasts and karyoplasts were shown to be of short-term survival (4-6) and expressed biological activities to nearly the same degree as intact cells (7, 8). Linder et al. reported that adult chicken erythrocyte nuclei can be reactivated in heterokaryons after cell fusion with mitomycin C-treated parental cells, such as myoblasts (9). Reactivation of erythrocyte nuclei in the mitomycin-C-treated cells resulted in the transcription and translation of adult chicken globin genes. It was also reported (7) that the surrounding cytoplasmic compartments gave regulatory signals to the nucleus. Our present as well as a previous study showed that irradiated splenic lymphocytes could become functional by fusion with lymphocyte nuclei. Such reconstituted lymphocytes were viable

for at least 72 h in vitro and could receive mitogenic signals with specific cell surface receptors, giving rise to the activated cytoplasmic factors in the cells. The present results suggest that different activated cytoplasmic factors might be produced in each lymphocyte subset, that is, some cytoplasmic factors raised in the Con A-stimulated T lymphocytes are effective on the T cell nuclei but not on the B cell nuclei. Cytoplasmic factors in the LPS-stimulated B lymphocytes, on the other hand, might be able to activate B cell nuclei but not T cell nuclei, suggesting that some restricted nucleocytoplasmic relationships might be involved in the activation of nuclei of mature T and B lymphocytes.

The nature of such a restricted nucleocytoplasmic relationship is not yet understood. Recently (10), we established a method to microinject macromolecules, such as protein molecules, into murine splenic lymphocytes by using polyethyleneglycol-mediated cell fusion. This method made it possible for us to transfer the cytoplasmic factors from one lymphocyte cell type into another. Very recently, using our microinjection technique, we found that microinjection of cytoplasmic protein(s) obtained from B cells of normal C3H/HeN mice could restore the LPS responsiveness of the B cells of LPS-nonresponsive mice, C3H/HeJ (manuscript in preparation). Combining the present methods with microinjection methods will be useful in clarifying the nature of the restricted nucleocytoplasmic relationship in T or B lymphocytes.

### Summary

Nuclei of murine T lymphocytes or B lymphocytes were purified and transferred into lethally irradiated whole spleen cells or B or T lymphocytes by means of polyethyleneglycol-mediated cell fusion. Transfer of lymphocyte nuclei could save the irradiated cells from cell death, and such reconstituted cells could respond to mitogens. The present study showed that nuclei of T cells could be activated in the concanavalin A-stimulated T cell cytoplasms but not in the lipopolysaccharide-stimulated B cell cytoplasms. On the other hand, nuclei of B cells were activated in the lipopolysaccharide-stimulated B cells but not in the concanavalin A-stimulated T cell cytoplasms. These data suggested that a specific interaction between cytoplasm and nucleus might exist in the activation of nuclei of each lymphocyte subset.

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